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Attachment B

Exhibit 4

Singer et al. (2004), " A MARCKS-related peptide blocks mucus hypersecretion in a mouse model of asthma" Nature Medicine 10(2):193-196.

nature medicine

A MARCKS-related peptide blocks mucus hypersecretion in a mouse model of asthma

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Mucus hypersecretion is a crucial feature of pulmonary diseases such as asthma, chronic bronchitis and cystic fibrosis. Despite much research, there is still no effective therapy for this condition. Recently, we showed that the myristoylated, alanine-rich C-kinase substrate (MARCKS) protein is required for mucus secretion by human bronchial epithelial cells in culture[1]. Having synthesized a peptide corresponding to the N-terminal domain of MARCKS, we now show that the intratracheal instillation of this peptide blocks mucus hypersecretion in a mouse model of asthma. A missense peptide with the same amino acid composition has no effect. Based on quantitative histochemical analysis of the mouse airways, the peptide seems to act by blocking mucus release from goblet cells, possibly by inhibiting the attachment of MARCKS to membranes of intracellular mucin granules. These results support a pivotal role for MARCKS protein, specifically its N-terminal region, in modulating this secretory process in mammalian airways. Intratracheal administration of this MARCKS-related peptide could therapeutically reduce mucus secretion in the airways of human patients with asthma, chronic bronchitis and cystic fibrosis.

Hypersecretion of mucin (the glycoprotein component of mucus) occurs in several respiratory diseases, including asthma, chronic bronchitis and cystic fibrosis, and is a risk factor for mortality in these patient groups. Currently, there are no therapies available to treat mucus hypersecretion. We recently showed that MARCKS, an 80- to 87-kDa protein that is a target for phosphorylation by protein kinase C, is required for mucin secretion in human bronchial epithelial cells in culture[1]. We also showed that an evolutionarily conserved, N-terminal, 24–amino acid fragment of MARCKS—myristoylated N-terminal sequence (MANS), or MARCKS-related peptide—inhibits mucin release in a concentration-dependent manner *in vitro*. In contrast, a missense control peptide with the same amino acid composition had no effect[1].

To test the effect of the MARCKS-related peptide *in vivo*, we used the ovalbumin (OVA)-sensitized mouse, a well-characterized model of allergic airway inflammation resembling human asthma[2–5]. To elicit mucin hypersecretion, we exposed OVA-sensitized mice of the BP2 strain to aerosolized methacholine for 90 s, 72 h after OVA challenge[4,5]. One group of mice was pretreated intratracheally (i.t.), 15 min before methacholine, with 50 μl MANS peptide in saline at 10, 100 or 140 μM (~1–15 mg /kg). A second group was pretreated with the missense peptide (random N-terminal sequence, or RNS) at 100 or 140 μM, and a third group was pretreated with saline alone. The amount of mucin released into tracheobronchial lavage fluid 30 min after methacholine exposure was measured by a mucin-specific ELISA, as described previously[3–5]. In mice treated with saline only, inhalation of methacholine caused an approximately fivefold increase in mucin secretion (Fig. 1a). Pretreatment with the MANS peptide resulted in a concentration-dependent decrease in this response, whereas pretreatment with the missense RNS peptide had no effect at either dose (Fig. 1a). In mice not exposed to methacholine, i.t. delivery of MANS peptide for 45 min decreased basal, constitutive levels of mucin secretion by 34 ± 7% and 69 ± 11% at 10 and 100 μM, respectively, compared with saline-treated mice. RNS peptide had no effect on basal mucin secretion. Histological examination of airways and analysis of bronchoalveolar lavage (BAL) fluid revealed no signs of injury or toxicity for up to 24 h after instillation of either peptide. FITC-conjugated MANS (100 μM) instilled i.t. into a mouse airway was detected within epithelial cells 15 min later, indicating intracellular localization.

To detect possible strain-specific effects of the peptides, we conducted similar studies in BALB/c mice. The results of these experiments were essentially the same as those observed in experiments with BP2 mice (Fig. 1b). The inhibitory effect of the MANS peptide was not restricted to a single secretory stimulus, as indicated by data showing inhibitory effects of MANS peptide on secretion induced by intraperitoneal (i.p.) delivery of 150 mg/kg pilocarpine (Fig. 1c).

To gain insight into the mode of action of MANS peptide, we examined the airways of OVA-exposed mice that had or had not received peptide treatment. Histochemical staining with periodic acid–Schiff (PAS) and hematoxylin revealed that airways exposed to OVA alone contained numerous mucin-filled goblet cells (Fig. 2a),

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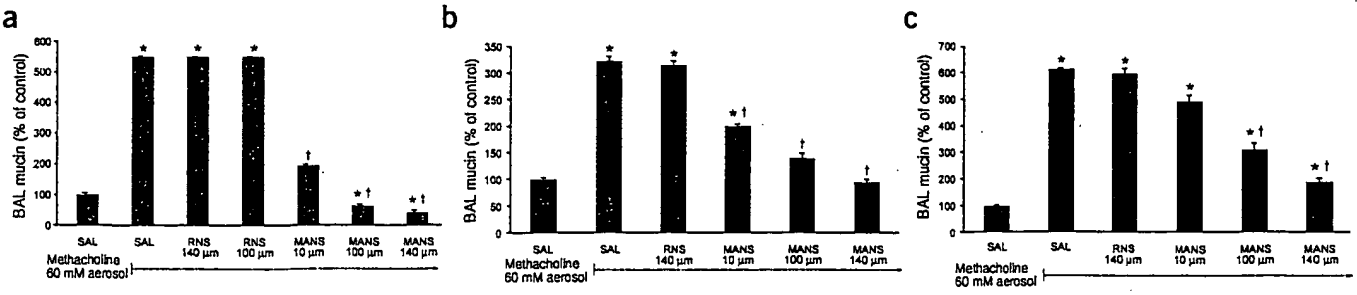

Figure 1 Effects of peptides on mucin secretion in mouse airways. (a) In BP2 mice challenged and sensitized with OVA, MANS peptide, but not missense RNS peptide, attenuates mucin secretion in response to methacholine, in a concentration-dependent manner. (b,c) In BALB/c mice sensitized and challenged with OVA, release of mucin into airways in response to inhaled aerosolized methacholine (b) or i.p. pilocarpine (c) is attenuated by pretreatment with MANS peptide, whereas RNS peptide does not affect mucin secretion. *, $P < 0.05$ compared with control; †, $P < 0.05$ compared with methacholine stimulation.

whereas upon methacholine exposure, PAS-positive material was lost from the airway epithelium and immunoreactive mucin appeared in the lavage fluid (Fig. 2b). Pretreatment with missense RNS peptide (Fig. 2c) had no effect, whereas pretreatment with MANS peptide (Fig. 2d–f) markedly reduced the effects of methacholine in a concentration-dependent manner. Quantitative morphometric analysis of these sections confirmed a concentration-dependent retention of mucin in airways of MANS-treated mice (Table 1).

The issue of how the MANS peptide interferes with mucin secretion has not yet been resolved. Studies of secretion using MARCKS knockout mice have not been done, as deleting the MARCKS gene causes CNS defects that result in death shortly after birth[6]. Work in a variety of isolated or cultured cell types however indicates that the phosphorylation state of MARCKS controls its affinity for actin filaments; it has been proposed that MARCKS-actin networks reduce the ability of secretory granules to penetrate the cortical cytoskeleton[7–12]. Supporting this, some studies have found that secretion can be attenuated by exposing cells to a peptide that blocks protein kinase C-mediated MARCKS phosphorylation[7,8].

This type of mechanism cannot however explain the results shown here, because mucin-secreting airway goblet cells lack a cortical actin ring, and because the N-terminal MANS peptide used in our studies does not interfere with MARCKS phosphorylation. Instead, the peptide used in our studies corresponds to the myristoylated N-terminal region of MARCKS, which regulates the interaction of MARCKS with membranes, and neither serves as a protein kinase C phosphorylation site nor binds or cross-links actin[13].

To account for the inhibitory effect of the MANS peptide in goblet cells, we propose a mechanism whereby MARCKS binds, at different sites, to secretory granule membranes and to the actin cytoskeleton. MARCKS would therefore serve as a physical link between the contractile cytoskeleton and mucin granules, and could have a role in guiding secretory granules to docking sites on the cell membrane. We have obtained biochemical evidence for such a mechanism in isolated human bronchial epithelial cells[1].

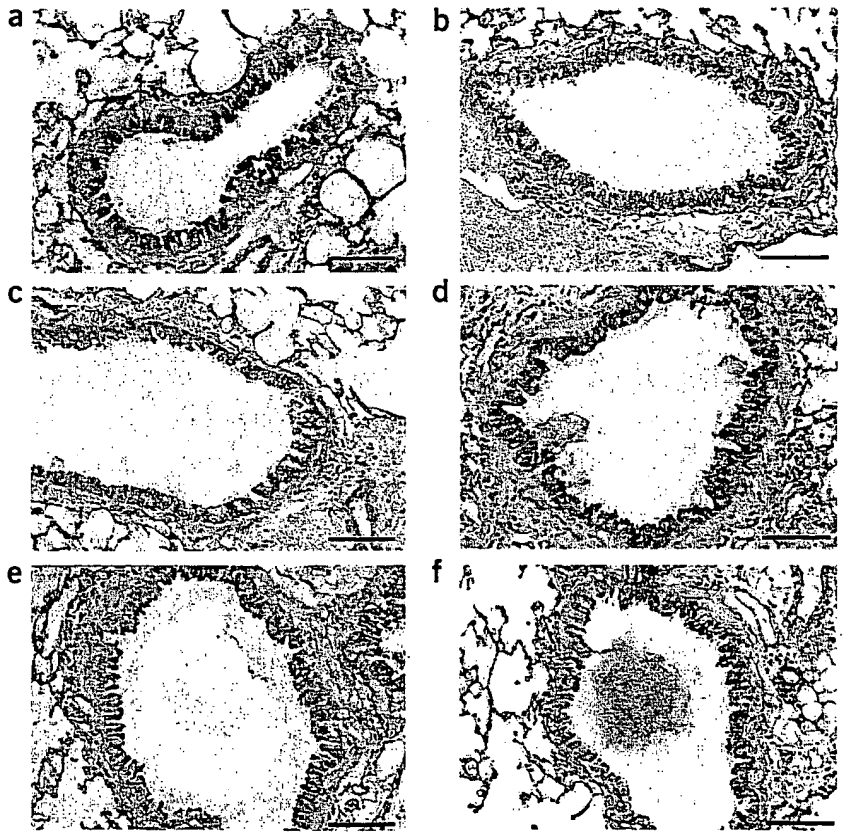

Figure 2 Representative PAS- and hematoxylin-stained sections of bronchi from sensitized and challenged BALB/c mice. (a) Mouse exposed to i.t. saline, followed by aerosolized saline. Goblet cells replete with PAS-positive intracellular mucin granules populated the epithelium. (b) Mouse exposed to i.t. saline, followed by aerosolized methacholine. Goblet cells released mucin granules; epithelium was mostly PAS-negative. (c) Mouse exposed to i.t. RNS peptide, followed by aerosolized methacholine. Epithelium was similar to that in b. (d–f) Mice exposed to i.t. MANS peptide at 140 µM (d), 100 µM (e) or 10 µM (f), followed by aerosolized methacholine. Mucin seemed to be retained in goblet cells after MANS treatment, whereas RNS peptide did not prevent methacholine-induced release of PAS-positive granules. Scale bars, 100 µm.

Here we used gold-labeled immunostaining for MARCKS to provide ultrastructural evidence of the association between MARCKS protein and membranes of mucin granules (Fig. 3a,b). MARCKS also bound membranes of mucin granules isolated from human bronchial epithelial cells; this binding was inhibited by MANS peptide (Fig. 3c). Because the MANS peptide used in these studies corresponds to the myristoylated N-terminus of MARCKS, it may be inhibiting endogenous MARCKS by competing for granule membrane binding. A sequence targeting MARCKS to specific membrane sites might reside in a region on the MARCKS protein between the myristoyl moiety and the phosphorylation-site domain[13], and might perhaps be related to the conserved multiple homology-2 (MH2) domain in this region[14].

In summary, we have shown that a peptide directed against the conserved N-terminal region of MARCKS protein inhibits mucin release *in vivo* when instilled i.t. into allergically inflamed mouse airways. The MANS peptide is the first example of a reagent with such properties. These findings lay the foundation for the development of new compounds that may find an important place in therapy for mucus hypersecretory disorders, such as those in patients with asthma, chronic bronchitis and cystic fibrosis.

METHODS

Ovalbumin-sensitized mouse model of mucus hypersecretion. All studies were approved by the Animal Care and Use Review Committee of the Institut Pasteur. Male BP2 or BALB/c mice (Centre d'Elevage R. Janvier), aged 6–8 weeks, were immunized subcutaneously with OVA (ICN Pharmaceuticals) as described previously[2]. Two weeks later, mice were anesthetized with 12% xylazine (20 mg/kg) and ketamine 500 (45 mg/kg) (Sigma), and challenged with 10 μg OVA in 50 μl of endotoxin-free 0.9% saline. OVA was administered under light anesthesia through a temporary cannula introduced into the trachea through the oral cavity.

Bronchoalveolar lavage. Mice were anesthetized i.p. with urethane (45 mg per 30 g body weight), and the trachea was incised and cannulated. BAL fluid was collected with 2.5 ml saline containing 0.005M EDTA, PMSF and DTT, all from Sigma.

Measurement of mucin secretion. Cell-free BAL fluid was assayed for secreted mucin using a previously described ELISA[4,5]. We used a mouse Muc5AC-specific antibody, raised against the peptide QTSSPNTGKTSTISTT from the mouse homolog of the *Muc5ac* gene, which recognizes lung, gastric and intestinal Muc5AC mucins[15].

Table 1 Morphometric analysis of mucin content in bronchi of BALB/c mice

	Mucin (mm^2 in airways)
Saline + MCH	0.01 ± 0.014**
OVA	1.81 ± 0.44**
OVA + MCH	0.07 ± 0.04
OVA + RNS (140 μM) + MCH	0.04 ± 0.01
OVA + MANS (140 μM) + MCH	1.44 ± 0.17**
OVA + MANS (100 μM) + MCH	0.40 ± 0.17*
OVA + MANS (10 μM) + MCH	0.14 ± 0.04

Sections from BALB/c mice, stained with PAS and hematoxylin, were analyzed for mucin content using an image analysis system. Results are mean ± s.d; $n = 25$ bronchi per group. MCH, methacholine. *, $P < 0.05$ compared with OVA + MCH; **, $P < 0.001$ compared with OVA + MCH.

Treatments. Seventy-two hours after OVA challenge, mice were instilled i.t. with a bolus of either saline, MANS peptide (identical to the first 24 amino acids of the N terminus of MARCKS; myristic acid–GAQFSKTAAKG EAAAERPGEAAVA) or missense control RNS peptide; myristic acid–GTA-PAAEGAGAEVKRASAEAKQAF), as described previously[1]. MANS or RNS peptides were administered at final concentrations of 10, 100 or 140 μM (~1–15 mg/kg). Mucin secretion was triggered 15 min later by administration of either methacholine, using a Buxco system nebulizer delivering aerosolized methacholine at 60 mM for 90 s, or i.p. injection of pilocarpine (150 mg/kg) for an additional 30 min before BAL and mucin measurement.

Histochemistry and quantitative morphometry. After the above exposures, lungs from selected BALB/c mice were flushed to remove blood, then inflated with OCT medium (Sakura Finetek) half-diluted in saline. Lungs were immersed in 10% phosphate-buffered formalin overnight at 4 °C, then embedded in paraffin. Five-micron-thick sections were stained with PAS and hematoxylin, and mucin retained in the epithelium in longitudinal sections of main-stem bronchi was quantified as described[4,16] using an image analysis system (Grastek Optilab software, version 2.1). We used five mice per treatment. For each mouse, we calculated the sum of the values of five fields per slide, for five slides, and converted the area from pixels to mm^2. All data were obtained in a blinded fashion at a magnification of ×200.

Ultrastructural immunohistochemistry. Trachea from BALB/c mice were immersion-fixed in 4% formaldehyde and 0.01% glutaraldehyde for 1h.

Figure 3 The association of MARCKS with mucin granule membranes is inhibited by MANS peptide. (a) Immunogold EM analysis of a goblet cell from mouse airway shows association of MARCKS with membranes (arrows) of mucin granules (mg). Scale bars, 300 nm. (b) Control for a, using gold-labeled preimmune IgG. (c) Immunoprecipitation of isolated mucin granule membranes from NHBE cells by gob-5-specific antibody. Lane 1, western blot showing detection of gob-5 in granule membranes; lanes 2–4, western blots showing MARCKS-specific staining of isolated mucin granule membranes (2, control cells; 3, granule membranes from cells exposed to 100 μM RNS; 4, greatly diminished staining of granule membranes from cells exposed to 100 μM MANS).

Sections were embedded in 3–4% agar, then dehydrated and embedded in LR White resin (Electron Microscopy Sciences). Ultrathin (80–90 nm) sections were blocked with 10% FBS for 15 min, then incubated overnight at 4 °C with an affinity-purified rabbit antibody to mouse MARCKS protein (Abcam; 3 μg/ml), in PBS containing 0.5% BSA. After washing, grids were incubated with 12-nm gold–labeled secondary antibody (donkey anti-rabbit; Jackson ImmunoResearch Laboratories) in PBS containing 0.5% BSA for 2 h, then poststained with uranyl acetate.

Isolation of mucin granules from normal human bronchial epithelial (NHBE) cells. Intact mucin granules were isolated by a modification of the Wu and Castle method[17]. NHBE cells in primary air-liquid interface culture were collected 14 d after reaching confluence, lysed via sonication and centrifuged at 600*g* for 10 min. Postnuclear supernatants were diluted with 1.9 volumes of 86% Percoll, 0.3 M sucrose, 5 mM MOPS, 1 mM EDTA and 0.2 μg/ml of DPPD (pH 6.8) before centrifugation at 17,000*g* for 30 min. Crude mucin granules collected from the bottom 0.4 ml of the gradient were diluted and pelleted at 2,000*g* for 15 min, before overnight incubation at 4 °C in PBS containing 0.3 M sucrose with Dynal beads coated with a monoclonal antibody to mouse $CLCA_3$ (gob-5; an integral component of mucin granule membranes[18]). After incubation, the antibody-granule complex was eluted, boiled with 2× Laemmli sample buffer and applied to SDS-PAGE before immunoblotting using MARCKS- and $CLCA_3$-specific antibodies.

For immunoblotting, proteins were transferred to a polyvinylidene fluoride membrane, rinsed, blocked in 5% nonfat dry milk for 1 h, and incubated with primary antibody and 1% BSA overnight at 4 °C. After washing, the membrane was incubated with secondary antibody for 1 h. Biotinylated secondary antibody (Jackson ImmunoResearch Laboratories) and VECTASTAIN ABC (Vector Laboratories) were used to detect MARCKS. Proteins were detected with an Amersham ECL kit, followed by exposure to Hyperfilm ECL (Amersham Biosciences).

Statistical analysis. Results are presented as mean ± s.d. ($n = 5–12$ for each point, for ELISAs). Significance levels were calculated using one-way ANOVA, followed by the Scheffe test, using SPSS 6.1 software. $P < 0.05$ was considered significant.

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COMPETING INTERESTS STATEMENT

The authors declare competing financial interests (see the *Nature Medicine* website for details).

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1. Li, Y., Martin, L.D., Spizz, G. & Adler, K.B. MARCKS protein is a key molecule regulating mucin secretion by human airway epithelial cells *in vitro*. *J. Biol. Chem.* **276**, 40982–40990 (2001).
2. Eum, S.Y., Haile, S., Huerre, M. & Vargaftig, B.B. Eosinophil recruitment into the respiratory epithelium following antigenic challenge in hyper-IgE mice is accompanied by interleukin-5-dependent bronchial hyperresponsiveness. *Proc. Natl. Acad. Sci. USA* **92**, 12290–12294 (1995).
3. Haile, S. *et al.* Mucous cell metaplasia and inflammatory cell recruitment are dissociated in allergic mice following antibody- and drug-dependent cell depletion in a murine model of asthma. *Am. J. Respir. Cell Mol. Biol.* **20**, 891–902 (1999).
4. Singer, M., Lefort, J. & Vargaftig, B.B. Granulocyte depletion and dexamethasone differentially modulate airway hyperreactivity, inflammation, mucus accumulation, and secretion induced by rmIL-13 or antigen. *Am. J. Respir. Cell Mol. Biol.* **26**, 74–84 (2002).
5. Vargaftig, B.B. & Singer, M. Leukotrienes, IL-13, and chemokines cooperate to induce BHR and mucus in allergic mouse lungs. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **284**, L260–L269 (2003).
6. Stumpo, D.J., Bock, C.B., Tuttle, J.S. & Blackshear, P.J. MARCKS deficiency in mice leads to abnormal brain development and perinatal death. *Proc. Nat. Acad. Sci. USA* **92**, 944–948 (1995).
7. Rose, S.D., Lejen, T., Zhang, L. & Trifaro, J.M. Chromaffin cell F-actin disassembly and potentiation of catecholamine release in response to protein kinase C activation by phorbol esters is mediated through myristoylated alanine-rich C kinase substrate phosphorylation. *J. Biol. Chem.* **276**, 36757–36763 (2001).
8. Elzagallaai, A., Rose, S.D. & Trifaro, J.M. Platelet secretion induced by phorbol ester stimulation is mediated through phosphorylation of MARCKS: a MARCKS-derived peptide blocks MARCKS phosphorylation and serotonin release without affecting pleckstrin phosphorylation. *Blood* **95**, 894–902 (2000).
9. Vaughan, P.F., Walker, J.H. & Peers, C. The regulation of neurotransmitter secretion by protein kinase C. *J. Mol. Neurobiol.* **18**, 125–155 (1998).
10. Trifaro, J., Rose, S.D., Lejen, T. & Elzagallaai, A. Two pathways control chromaffin cell cortical F-actin dynamics during exocytosis. *Biochimie* **82**, 339–352 (2000).
11. Salli, U., Supanic, S. & Stormshak, F. Phosphorylation of myristoylated alanine rich C kinase substrate (MARCKS) protein is associated with bovine luteal oxytocin exocytosis. *Biol. Reprod.* **63**, 12–20 (2000).
12. Hartness, M.E., Wade, J.A., Walker, J.H. & Vaughan, P.F. Overexpression of the myristoylated alanine-rich C kinase substrate decreases uptake and K^+-evoked release of noradrenaline in the human neuroblastoma SH-SY5Y. *Eur. J. Neurosci.* **13**, 925–934 (2001).
13. Seykora, J.T., Myatt, M.M., Allen, L.A.H., Ravetch, J.V. & Aderem, A. Molecular determinants of the myristoyl-electrostatic switch of MARCKS. *J. Biol. Chem.* **271**, 18797–18802 (1996).
14. Spizz, G. & Blackshear, P.J. Identification and characterization of cathepsin B as the cellular MARCKS cleaving enzyme. *J. Biol. Chem.* **272**, 23833–23842 (1997).
15. Shekels, L.L. *et al.* Mouse gastric mucin: cloning and chromosomal localization. *Biochem. J.* **311**, 775–785 (1995).
16. Vargaftig, B.B. & Singer, M. Leukotrienes mediate part of Ova-induced lung effects in mice via EGFR. *Am. J. Physiol. Lung Cell Mol. Physiol.* **285**, L808–L818 (2003).
17. Wu, T.T. & Castle, J.D. Evidence for colocalization and interaction between 37 and 39 kDa isoforms of secretory carrier membrane proteins (SCAMPs). *J. Cell Sci.* **110**, 1533–1541 (1997).
18. Leverkoehne, I. & Gruber, A.D. The murine mCLCA3(alias gob-5) protein is located in the mucin granule membranes of intestinal, respiratory and uterine goblet cells. *J. Histochem. Cytochem.* **50**, 829–838 (2002).